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CHARAKTERISIERUNG EINES BEI IMMUNSUPPRESSION FREIGESETZTEN STIMULATIONSFAKTORS

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Characterization of a Stimulating Factor Released by Immunosuppression

Charakterisierung eines durch Immunsuppression freigesetzten Stimulationsfaktors

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With 2 figures

Abstract

To get more information about the mechanism of stimulation of the immune response during immunosuppression under certain conditions, stimulating factor (SF) was collected 48 hours after treatment of NMRI-mice with cyclophosphamide. SF was characterized by different steps of separation using centrifugation, dialysis and salt-fractionation. For demonstration of the SF animals were sensitized with SRBC and given different substrates separately. The stimulation of the antibody-production was demonstrated on the cellular level using the plaque-technique. SF was detected by comparing the counts of plaque-forming cells in the spleen of treated mice compared with controls merely sensitized or given the same substrate of normal animals. In this way it was shown that the SF is a soluble factor in the serum with a molecular weight of more than 20,000, but that it is no immunoglobulin.

The manipulation of the immune response is a requirement with respect to the disorders found in various diseases. The most used treatment is the suppression of pathogenic immune reactions. Although the mode of action of the applied substances is well known, there are effects that are not yet explained. One of them is the stimulation of the immune response by immunosuppressive agents under certain conditions. This was demonstrated with substances of different mode of action (2, 3) as well as with X-rays (4) and even with substances where the immunosuppressive action was only one of several effects (8). It has been supposed that fragments of disintegrated cells or liberation of cells from places of recruitment cause this contrary effect. A hypothetical feedback system causing that effect was denied in further experiments (7). That means that a stimulatory factor is set free during this phase. It was of interest to characterize that factor using different techniques of separation. For demonstration of the stimulating effect

the cellular antibody-formation was measured by Jerne's plaque-technique. The conditions refer to former experiments dealing with the stimulation of antibody-synthesis with immunosuppressive agents (1).

Materials and Methods

Animals: The experiments were performed with mice of the strain NMRI. Their weight was about 25 g, their age 3–6 months. In each series animals of the same sex were used. They were fed with pellets and water.

Chemical immunosuppression: Each animal was given intraperitoneally (i.p.) 2 mg cyclophosphamid (Cph) in an isotonic and isoionic solution in one dose.

Demonstration of the stimulating factor (SF): The SF was demonstrated by the humoral immune response basing on the antibody-formation on the cellular level. For that reason animals were sensitized by i.p. application of 4×10^8 sheep red blood cells (SRBC). After an interval of 2 hours animals were inoculated with different substrates that were suggested to contain the SF (the mode of collection is described in the following section). 7 days later the animals were sacrificed by cervical dislocation. The spleen was removed and separated from fat and connective tissue. To demonstrate single antibody-producing cells the plaque-technique was performed in the usual way (5, 6). The direct method detected 19S-immunoglobulin-producing direct plaque-forming cells (DPFC), the indirect method 7S-immunoglobulin producing indirect PFC (IPFC).

Controls were on the one hand animals sensitized with SRBC and inoculated in the same manner but with substrates of untreated animals, on the other hand animals only sensitized with SRBC.

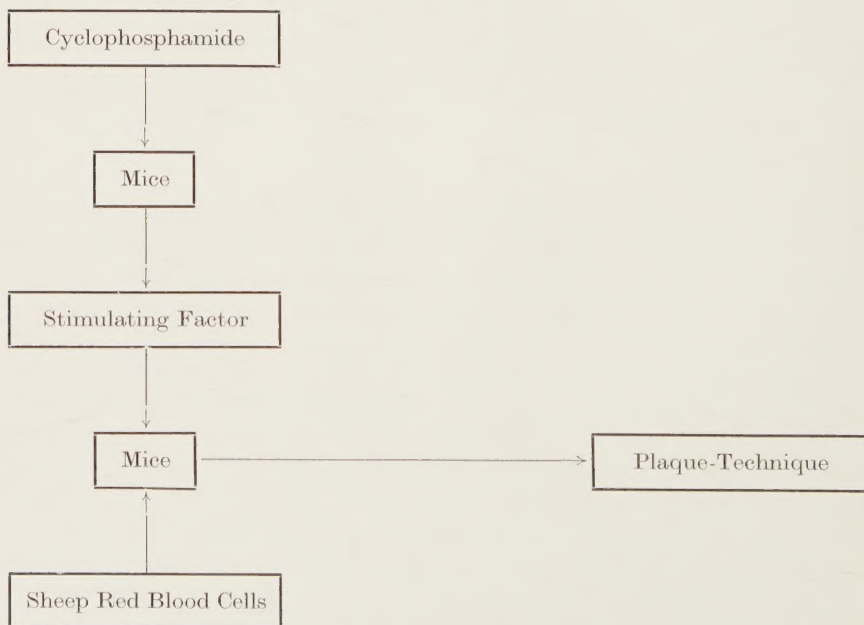


Fig. 1. Experimental plan for demonstration of the stimulating factor (SF)

The effect of the SF was calculated by comparison of the antibody-formation in normal and in treated animals. In each experiment 6 animals were separately tested counting several dishes. The experimental plan is shown in Figure 1.

Isolation and characterization of the SF: The animals were sacrificed 48 hours after treatment with Cph because the SF is set free at that time. They were instantly used for further characterization of the SF. This was performed in the following way:

- a) To show whether the SF is located in cells or in serum, spleen was removed and blood was collected. Single spleen cells were isolated by pressing the organ through a steel mesh. Serum was prepared by centrifugation of the blood.
- b) To show whether the SF is identical with soluble factors or with circulating particles, sera of treated animals were centrifuged at 100,000 g for 45 minutes and supernatant was separated from sediment.

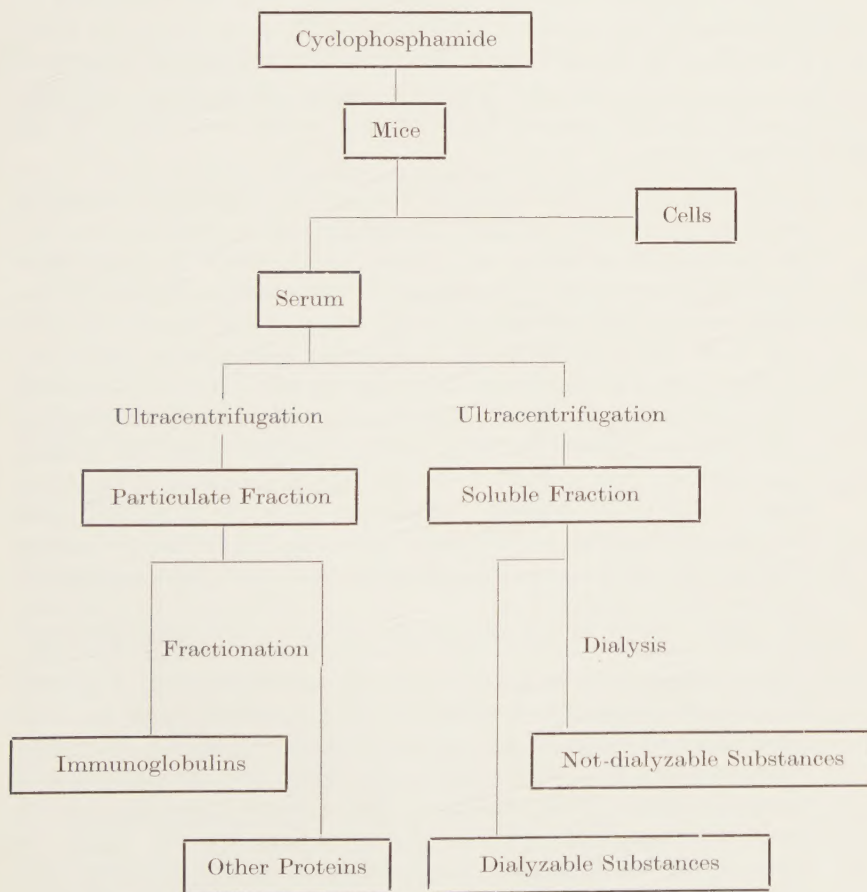


Fig. 2. Characterization of the stimulating factor (SF)

- c) To get some information about the molecular weight of the SF sera were dialyzed against sodium using a membrane with pores of 15–20 Å in diameter, i.e. only molecules of a weight not more than 10,000–20,000 could pass.
- d) To show wheather the SF is identical with antibodies, immunoglobulins of the sera of treated animals were separated from the other soluble factors by fractionation using ammoniumsulphate.

To get enough substrate containing SF the material of several treated animals was pooled: for demonstration of the SF the equivalence of 10^8 spleen cells respectively 10 ml serum was inoculated into sensitized animals. In the same manner substrate was obtained from untreated animals for controls.

A flow sheet of the preparation of the SF is given in Figure 2.

Results

A primary immune response against SRBC was demonstrated in each experiment. The counts of DPFC and IPFC were nearly the same as it is usually seen 1 week after sensitization. The spleens of sensitized animals were slightly enlarged in comparison to not sensitized animals. There was no malaise caused by treatment or by inoculation of the different substrates.

- a) Identification of the SF in the serum: In the 1st series it could be demonstrated that the serum was containing the SF, for inoculation of serum of treated mice caused a stimulation from 250% up to 740% in respect to untreated animals whereas inoculation of normal serum could increase the counts of PFC only from 110% up to 180%. Comparing both groups, the stimulating effect was more than doubling the PFC. In contrast, there was no stimulation seen in animals that were applicated spleen cells of treated mice (from 50%–190%). In a 2nd series that finding was verified: inoculation of normal serum caused a stimulation from 178%–183% whereas inoculation of serum of treated mice stimulated from 286%–338% once more nearly doubling the counts of PFC. The differences are significant ($p < 0.05$). The results are summarized in Table 1.
- b) Identification of the SF in soluble factors: After fractionation of small particles from soluble substances of serum of treated animals and separate inoculation into sensitized mice in both series a stimulation of the immune response within the same range was seen, i.e. between 172% and 283%. In contrast, serum of normal animals could stimulate the immune response only to 120% at most. This experiment shows that the SF cannot be spun down, but that it is dispersed all over the fractions. The difference is significant ($p < 0.05$). The results are summarized in Table 2.

Tab. 1. Identification of the stimulating factor in the serum (separation of serum and cells)

Application of	DPFC		IPFC	
	Series I	Series II	Series I	Series II
—	177 ± 37.2 1.0	134 ± 19.5 1.0	65 ± 17.1 1.0	147 ± 35.9 1.0
Serum of normal mice	210 ± 48.6 1.1	238 ± 49.7 1.78	117 ± 12.8 1.8	224 ± 44.8 1.53
Spleen cells of normal mice	252 ± 125.2 1.4		197 ± 125.0 3.0	
Serum of treated mice	512 ± 113.0 2.9	383 ± 45.9 2.86	465 ± 64.3 7.4	496 ± 78.6 3.38
Spleen cells of treated mice	90 ± 53.2 0.5		122 ± 58.4 1.9	

Counts of PFC and standard deviation are summarized above, the relation to controls (= 1.0) below.

Statistical differences ($p < 0.05$) according to Student's T-test are marked.

Tab. 2. Identification of the stimulating factor in the soluble fraction (ultracentrifugation)

Application of	DPFC	IPFC
—	124 ± 13.1 1.0	177 ± 28.3 1.0
Normal serum	148 ± 17.8 1.20	205 ± 21.0 1.16
Soluble fraction of treated mice	312 ± 15.0 2.52	304 ± 25.2 1.72
Particulate and soluble fraction of treated mice	260 ± 46.4 2.10	500 ± 30.2 2.83

Counts of PFC and standard deviation are summarized above, the relation to controls (= 1.0) below.

Statistical differences ($p < 0.05$) according to Student's T-test are marked.

e) Size of the SF: By use of a membrane letting pass through molecules smaller than 10 Å according to a molecular weight of 20,000 at most those substances were separated from smaller serum-factors. It was shown that after dialysis the stimulating effect was as high (184%–186%) as before (188%–202%) demonstrating that the SF is not dialyzable. The counts of PFC in controls inoculated with normal serum varied from 97%–155%. The difference is significant ($p < 0.05$). The results are summarized in Table 3.

Tab. 3. Size of the stimulating factor (dialysis)

Application of	DPFC	IPFC
—	165 ± 6.0 1.0	145 ± 34.7 1.0
Full serum of normal mice	224 ± 20.9 1.36	224 ± 21.3 1.55
Serum of normal mice after dialysis	160 ± 13.8 0.97	217 ± 10.1 1.50
Full serum of treated mice	310 ± 19.6 1.88	292 ± 17.8 2.02
Serum of treated mice after dialysis	306 ± 20.0 1.86	266 ± 14.7 1.84

Counts of PFC and standard deviation are summarized above, the relation to controls (= 1.0) below.

Statistical differences ($p < 0.05$) according to Student's T-test are marked.

d) Exclusion of the SF from immunoglobulins: By separation of the immunoglobulins from other serum-factors it could be shown that the SF is not belonging to the immunoglobulins: inoculation of gammaglobulin of normal as well as of treated animals did not influence the immune response expressing in the counts of PFC between 70 and 109%. Application of the remaining fraction after treatment with Cph, however, caused an increase of the

Tab. 4. Exclusion of the stimulating factor from immunoglobulins (fractionation)

Application of	DPFC		IPFC	
	Series I	Series II	Series I	Series II
—	149 ± 20.5 1.0	80 ± 18.3 1.0	186 ± 26.1 1.0	129 ± 12.0 1.0
Immunoglobulins of normal mice	122 ± 12.7 0.8	93 ± 18.6 1.16	138 ± 16.8 0.7	141 ± 14.4 1.09
Serum without immunoglobulins of normal mice	155 ± 20.3 1.06	68 ± 17.2 0.9	230 ± 18.1 1.2	88 ± 14.8 0.6
Immunoglobulins of treated mice	161 ± 15.9 1.08	69 ± 15.5 0.8	200 ± 18.1 1.07	88 ± 16.9 0.6
Serum without immunoglobulins of treated mice	235 ± 18.6 1.5	141 ± 24.7 1.7	318 ± 23.6 1.7	242 ± 17.7 1.7

Counts of PFC and standard deviation are summarized above, the relation to controls (= 1.0) below.

Statistical differences ($p < 0.05$) according to Student's T-test are marked.

counts of PFC to 170% whilst the same substrate of normal mice had a rate of counts between 60% and 120%. The difference is significant ($p < 0.05$). The results are summarized in Table 4.

Discussion

Before interpreting the results some peculiarities of the immune system have to be pointed out. The immune response as a matter of different steps is also susceptible to nonspecific factors. Thus a true influence on the antibody-formation can only be verified considering the results in both results, i.e. in the animals merely sensitized and in those that were given substrates originating from normal mice. Moreover, a true influence has to be demonstrated constantly. Considering those facts, a nonspecific factor was seen in serum of normal mice and in the spleen-cells of treated animals. This was, however, not significant, because these results were not constant, whereas in other groups the same influence was demonstrated in each single experiment.

As seen in former experiments, the primary immune response has been stimulated by a particular mode of application of immunosuppressive drugs. Most important is the fact that the enhancing effect is not achieved by living cells. This refutes the supposition that the initial stimulation during treatment with cytostatic drugs is due to a release of cells out of places of origination into the blood. Moreover, it is striking that the SF is not identical with fragments of cells, for this interpretation was deduced from a direct cytotoxic effect of those drugs.

Since the SF is a soluble substance it is of interest to which category of serum factors it is belonging. The experiments show that its molecular weight is more than 20,000 but that it is no immunoglobulin. The last detail is most important, for there is no specific factor responsible for the stimulation. Therefore also a complex formed by antigen and antibodies is not involved in the stimulation of the immune response. However, another fact excluded that speculation already: the production of SF was performed in unsensitized mice that had no contact with SRBC. Considering these data there is a curious situation, for the SF can be defined only by summing up qualities that are not demonstrated: it is not identical with cells or cell-fragments. Belonging to the soluble fraction of the serum its molecular weight is more than 20,000. There is no connexion between the SF and the immunoglobulins. These qualities of the SF are remarkable and in consent with other mitogenic substances that are stimulating the immune response.

It must be assumed that not only Cph but also other immunosuppressive drugs can release the SF. Further investigation has to add new data about the SF. In this way not only unknown direct effects of immunosuppressive drugs will be detected but also mechanisms to stimulate the immune response.

Zusammenfassung

Charakterisierung eines durch Immunsuppression freigesetzten Stimulationsfaktors

Um weitere Einzelheiten über einen unter besonderen Bedingungen während der Immunsuppression freigesetzten Stimulationsfaktor (SF) zu erfahren, wurden NMRI-Mäuse mit Cyclophosphamid behandelt und der SF 48 Stunden später gewonnen. Er wurde durch verschiedene Trennverfahren, Zentrifugation, Dialyse und Salzfällung, weiter charakterisiert. Zum Nachweis wurden die Versuchstiere mit Hammelerythrozyten sensibilisiert und die verschiedenen Substrate getrennt verabreicht. Die Stimulation der Antikörpersynthese wurde mit der Plaque-Technik nachgewiesen, wobei unbehandelte sowie mit denselben Substraten von Normaltieren behandelte Mäuse als Kontrollen dienten. Hierdurch wurde gezeigt, daß der SF einen löslichen Serumfaktor darstellt, dessen Molekulargewicht 20000 überschreitet und der nicht zu den Immunglobulinen gehört.

References

1. BAENKLER, H. W., and F. SCHEIFFARTH: Stimulation of antibody-synthesis with immunosuppressive drugs in parabionts. *J. Med.* **2**, 143-148 (1971).
2. BERENBAUM, M. C., and I. N. BROWN: Dose-response relationships for agents inhibiting the immune response. *Immunology* **7**, 65-71 (1964).
3. CHANMOUGAN, D., and R. S. SCHWARTZ: Enhancement of antibody synthesis by 6-mercaptopurine. *J. exp. Med.* **124**, 363-378 (1966).
4. DIXON, F. J., and P. J. MCCOHANEY: Enhancement of antibody-formation by whole body X-irradiation. *J. exp. Med.* **115**, 833-844 (1963).
5. DRESSER, D. W., and H. H. WORTIS: Use of an antiglobulin serum to detect cells producing antibody with low hemolytic efficiency. *Nature (London)* **208**, 859-861 (1965).
6. JERNE, N. K., and A. A. NORDIN: Plaque formation in agar by single antibody producing cells. *Science* **140**, 405 (1963).
7. SAHAR, K., and R. S. SCHWARTZ: Inhibition of 19S-antibody synthesis by 7S-antibody. *Science* **145**, 395-397 (1964).
8. SCHEIFFARTH, F., H. W. BAENKLER and S. PFISTER: The influence of gold on the kinetics of plaque forming cells. *Int. Arch. Allergy* **40**, 117-123 (1971).

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LEBENS LAUF

Am 11. Oktober 1944 wurde ich, Alois Zehentner, als zweites Kind des Försters und Holzhändlers Wolfgang Zehentner und seiner Ehefrau Maria, geb. Schmid, in Velden/Vils geboren.

Von 1950 - 1956 besuchte ich die Volksschule in Neufraunhofen. Im September 1956 trat ich in das Hans-Carossa-Gymnasium in Landshut ein, an dem ich im Juli 1965 das Abitur bestand.

Im Oktober 1965 wurde ich zur Bundeswehr einberufen. Zwei Jahre später, im September 1967, wurde ich als Leutnant der Reserve entlassen.

Das Medizinstudium begann ich im Wintersemester desselben Jahres an der Friedrich-Alexander-Universität Erlangen. Im Oktober 1968 bestand ich hier das Vorphysikum, im April 1970 das Physikum und werde im Juli 1973 das Staatsexamen ablegen.

